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Association Between *ApoE* Gene Polymorphism and Serum Lipid Profile in Coronary Heart Disease and Healthy Subjects

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of university of other institute, except where due acknowledgement has been made in the text.

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Abstract

Apolipoprotein E (*ApoE*) plays a role in the regulation of the lipid metabolism of humans. *ApoE*, 229 amino acid polypeptide, is classified into three major isoforms (E2, E3, E4) according to the differences of amino acid in positions 112 and 158. In the normal population *ApoE3* isoform is the most prevalent and *ApoE2* or *E4* are frequently associated with hyperlipoproteinemia. The objective of this study was to investigate the relationship between *ApoE* gene polymorphism and serum lipid profile and coronary heart disease (CHD) in Gaza Strip. Methods: Polymerase chain reaction-restricted fragments length polymorphism (PCR-RFLP) was used to determine *ApoE* genotypes using the two restrictionenzymes; AflIII (A) and HaeII (H). Our study population consisted of 137 subjects including 69 cases (45 male, 24 female) CHD and 68 (33 male and 35 female) healthy subjects. Two blood samples were collected from each subject one was whole blood and the other was serum after fasting for 12-16 hours. Results: the ApoE3/E3 genotype was the most common in the control and the CHD groups. ApoE2/E3 and *ApoE4/E3* were the next most common genotypes. The frequencies of *ApoE* alleles in the CHD subjects were: 0.826 for the E3, 0.137 for the E4 and 0.0362 for the E2. These frequencies are comparable to those found in the control group which were: 0.875 for the E3, 0.073 for the E4 and 0.0515 for the E2. No statistically significant differences in *ApoE* genotypes were found between the patients and the control groups. Moreover, there was no significant difference between the mean of triglyceride and HDL among the different *ApoE* genotypes. However, there was a significant difference in the mean of LDL and *ApoE* genotypes where the mean of LDL was (218.17mg\dl) in *ApoE4, (*149.67mg\dl) in *ApoE2* and (184.52mg\dl) in *ApoE3*. A significant difference was also evident between the mean of LDL levels in the CHD and the control group where the mean of LDL was (126mg\dl) in CHD and (111.47mg\dl) in the control group. Our study indicated that there was no significant difference between the mean of cholesterol and triglyceride levels

of the CHD and the control groups. To our knowledge, this is the first study in Gaza Strip investigating the relation between ApoE genotypes and CHD. Further investigations are needed to link other genetic factors to CHD.

Key words

Apolipoprotien E; PCR-RFLP; CHD; lipid profile; Gene polymorphism.

مدي الارتباط بين الأشكال المختلفة للجين (ApoE) ومستوي الدهون في كل من مرضي القلب و الأشخاص الأصحاء

الملخص:

يلعب (ApoE) دوراً مهم في تنظيم أيض الدهون داخل جسم الإنسان ويتكون هذا البروتين من 229 حمض أميني ،وينقسم إلي ثلاثة أنواع وهي (E3,E4,E2) وذلك حسب اختلاف الحمض الاميني في الموقع 158و112 .في أغلب المجتمعات وجد أن النوع E3 هو الأكثر شيوعاً بينما E4,E2 تتواجد بنسبة أقل. تهدف هذه الدراسة إلي التعرف علي العلاقة مابين أشكال الجين (ApoE) وأمراض القلب ومستوي الدهون في قطاع غزة حيث أجريت الدراسة في الفترة من بداية شهر يونيو إلي نهاية شهر ديسمبر 2005 وقد اشتملت الدراسة علي 137 عينة منهم 69 عينة لمرضي القلب (45 رجل و 24 سيدة) و68 عينة لأشخاص أصحاء (33 رجل و35 سيدة) وهي العينة الضابطة.

تم جمع العينات من كل من مجمع ناصر ومستشفي دار الشفاء والعينة هي عبارة عن جزأين جزء هو دم كامل والجزء الأخر هو مصل وذلك بعد صوم 12-16 ساعة وتم تحليل العينات وتم استخدام المصل لقياس الدهون في الدم أما الدم الكامل فقد استخدم لعزل ال DNA لمعرفة شكل الجين عن طريق استخدام تقنية إلPCR-RFLP.

وجدت الدراسة أن الشكل الجينيApoE3 هو الأكثر شيوعاً في كل من العينة الضابطة وعينة مرضي القلب أما النوعين الآخرين فكانا أقل. أوضحت الدراسة أن نسبة الأليل في مرضي القلب هي: ApoE3= 0.826، ApoE4 = 0.137، ApoE2 = 0.036 بينما كانت في العينة الضابطة كالتالي: ApoE3= 0.875 ، ApoE4 = 0.073، ApoE2 = 0.515 . كما أوضحت الدراسة أنه لا يوجد أي دلالة إحصائية تؤكد ارتباط مباشر بين أي من أشكال الجين بمرض القلب.وجدت أيضاً الدراسة أن الأشخاص الذين يملكون الأليل E4 كانت نسبة متوسط الكلسترول عندهم هي (218.17mg\dl)أي أنها اعلي من الأشخاص الذي يملكون الأليل E2 والذين كان متوسط الكلسترول لديهم منخفض و هو (149.67mg\dl) بينما في الأشخاص الذين يملكون E3 كان معتدلاً وهو (184.52mg\dl) وكان هذا ذو دلالة إحصائية حيث أن قيمة pكانت أقل من 0.05 . كما أوضحت الدراسة أن متوسط الكلسترول منخفض الكثافة كان مرتفعاً أيضا في الأشخاص اللذين يملكون الأليل E4 ومنخفض عند الأشخاص الذي يملكون الأليل E 2 وكان هذا ذو دلالة إحصائية حيث قيمة p كانت أقل من 0.05 أما بالنسبة للكلسترول عال الكثافة والشحوم الثلاثية وجد أن الاختلاف ليس ذو دلالة إحصائية.

وجدت الدراسة أن متوسط الكلسترول منخفض الكثافة مرتفعاً عند مرضي القلب حيث كان (126mg\dl) بينما منخفضاً عند الأشخاص الأصحاء وكان المتوسط (111.47mg\dl) وكان ذلك ذو دلالة إحصائية أما بالنسبة للكلسترول و الشحوم الثلاثية وجد أن الاختلاف ليس ذو دلالة إحصائية

توصي الدراسة بإجراء المزيد من الأبحاث علي أشكال الجينات الأخرى التي لها علاقة بتنظيم الدهون وأمراض القلب .

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ABBREVIATIONS

µl	Micro Liter
ABCA1	ATP-binding cassette protein 1
Apo E	Apolipoprotein E
ApoA1	apolipoprotein A1
Arg	Arginine
BD	Blood Donors
BMI	Body Mass Index
bp	Base Pair
BSA	Bovine serum Albumin
CAD	Coronary Artery Disease
CETP	cholesterol-ester transfer protein
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
Cys	Cysteine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EL	endothelial lipase
ERF	Established Risk Factor
HDL	High Density Lipoprotein
HL	hepatic lipase
IDL	Intermediated low density lipoprotien
LCAT	lecithin–cholesterol acetyl transferase
LDL	Low Density Lipoproteins
LDLR	low density lipoprotein receptor
LPL	lipoprotein lipase
mg	Milli gram
MI	Myocardial Infarction
O.D	Optical Density
O.R	Odds Ratio
PCR	Polymerase Chain Reaction
PLTP	phospholipid transfer protein
RFLP	Restriction Fragment Length Polymorphism
RP	Retinyl Palmitate
RRs	Relative Risks
SfA	Saturated Fatty Acid
SR-BI	scavenger receptor class B type I
ST	Standard
TC	Total Cholesterol
TG	Triglyceride
VLD L	Very Low Density Lipoprotein

To my husband who suffered a lot in helping me accomplishing this work

To my sons

Tarak, Ref'at, and Abdullah

To my Beloved and kind parents

To my father-in-low and mother-in-low

And

For my brother's soul; the martyr Ref'at.
I am also dedicating this humble work to all mankind hoping that it will be of a benefit to all.

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CHAPTER 1
INTRODUCTION

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INTRODUCTION

1.1. Background

The apolipoprotein (*Apo*) genes play a central and pervasive role in lipid metabolism by maintaining structural integrity of lipoproteins, acting as cofactors for lipid-processing enzymes, and serving as ligand for lipoprotein receptors. Polymorphisms of these genes may alter lipid levels in individuals, which may predispose to atherosclerosis. If dysfunctional alleles that predispose to atherogenic lipid alterations can be identified, screening for the presence of these alleles may identify a substantial proportion of high-risk individuals. Appropriate monitoring of these individuals, in conjunction with targeted intervention, could then delay or avert the onset of atherosclerosis **(1).**

The development of atherosclerosis is a complex process influenced by a network of risk factors, such as hypertension, hyperlipidemia, smoking, and genetic predisposition **(2).**

ApoE gene is located on the long (q) arm of chromosome 19 and codes for a protein that exists in several different forms. ApoE polypeptide is a polymorphic protein consisting of 299 amino acids long chain **(3).** ApoE, which is synthesized primarily in the liver and brain, is one of the five main types of blood lipoproteins (A–E) and is a major component of specific lipoproteins called very low-density lipoproteins (VLDLs). A major function of VLDLs is to remove excess cholesterol from the blood and carry it to receptors on the surface of liver cells for processing. ApoE helps in transporting lipids from one place to another and facilitates the clearing of dietary fats, such as triglycerides (TG), from the blood. Maintaining normal levels of cholesterol is essential for the prevention of cardiovascular diseases, including heart attacks and strokes. Defects in the ApoE protein could diminish its ability to bind to the receptors, which then leads to an elevated blood cholesterol level **(4).**

ApoE polymorphisms have been associated with variations in blood cholesterol level and with the risk of atherosclerosis and premature cardiovascular diseases **(5).**

ApoE is a glycoprotein that is a constituent of chylomicrons, VLDLs, intermediate density lipoprotein (IDLs), and high density lipoprotein (HDLs) and is a ligand for the receptor-mediated hepatic uptake of chylomicron and VLDL remnant particles **(6).** ApoE regulates the metabolism of lipoproteins. The gene for *Apo E* is polymorphic. It has three common alleles, E2, E3, and E4, which code for three major isoforms, resulting in six common genotypes. ApoE facilitates the binding of triglyceride-rich lipoprotein remnants to receptors that determine their clearance. The isoforms vary in their receptor-binding activity, with *ApoE4* having the greatest receptor binding and *ApoE2* having a decreased affinity. Individuals with *ApoE2* have higher levels of triglycerides, and *ApoE2* homozygotes have greatly increased concentrations of remnants, which frequently results in a form of dyslipidemia called type III or dysbetalipoproteinemia. Individuals with E4, conversely, tend to have higher concentrations of LDL cholesterol; this increase is due, in part, to more efficient absorption of dietary cholesterol and perhaps down regulation of the LDL receptor. Thus*, ApoE* is an important candidate gene for cardiovascular disease **(7).**

The *ApoE2* isoform has been shown both in vitro and in vivo to be defective in mediating the clearance of remnant lipoproteins by hepatic receptors. Some *ApoE2/2* homozygotes develop type III hyperlipoproteinemia, which is a distinctive genetic disorder of lipoprotein metabolism characterized by the clinically significant accumulation of remnant lipoproteins due to defective hepatic removal of these lipoproteins. These patients have both increased cholesterol and TG levels. The remnant lipoprotein, also known as ß-VLDL because of its ß-electrophoretic mobility, is the major abnormal lipoprotein in type III hyperlipoproteinemia. In addition, the ß-VLDL is enriched in cholesterol, as reflected by a ratio of VLDL cholesterol to plasma TG of more than 0.3.

Patients with type III hyperlipoproteinemia are predisposed to the development of premature atherosclerosis **(3).**

Approximately 1% of individuals in the general population are *ApoE2/2* homozygotes. However, only a small percentage (2% to 5%) of these *ApoE2/2* homozygotes develop type III hyperlipoproteinemia. **Utermann et al (1979)** suggested that homozygosity for *ApoE2* is a necessary but insufficient genetic influence and that additional genetic or environmental factors are required for the expression of type III hyperlipoproteinemia in *ApoE2/2* homozygotes. Until 1979, no other abnormalities in other genes have been identified **(8).** High levels of total and LDL cholesterol and low levels of HDL cholesterol predispose to the development of atherosclerosis. Serum levels of these lipids are partly determined by the *ApoE* genotype. The *ApoE* gene has 3 common alleles, *ApoE*2, *ApoE*3, and *ApoE*4, which fully determine the protein isoforms *ApoE*2, *ApoE*3, and *ApoE*4, respectively, and partly determine the level of ApoE protein in serum. Compared with *ApoE*3 homozygotes, the most common genotype, *Apo*E2, is associated with lower levels of total and LDL cholesterol and with higher levels of HDL cholesterol, while *ApoE*4 has opposite effects. ApoE plays a pivotal role in the transport of lipoproteins and is involved in numerous processes in the arterial wall **(9).**

Risk of atherosclerosis and coronary heart disease in middle age appears to be higher in the presence of the *ApoE4* allele, possibly because of altered lipid metabolism. Not all studies, especially those of elderly subjects or survivors of heart attack, however, find an association between *ApoE4* and coronary heart disease **(10).**

The ApoE4 allele (Arg-112 and Arg-158) has been linked to atherosclerosis. Individuals with *E4*/*E4* genotype are at a higher risk of developing the disease **(4).**

1.2. Objectives of the Study

- To investigate the association between *ApoE* gene polymorphism and coronary heart disease (CHD).
- To evaluate the effect of *ApoE* gene polymorphism on serum lipid levels.
- To investigate the association between serum lipid levels and coronary heart disease (CHD).
- To investigate the association between smoking, age and (CHD).

CHAPTER 2
LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1. Background

Cardiovascular diseases, including CHD and stroke, are the most important causes of mortality and morbidity in Western countries. Several studies have indicated that the classic cardiovascular risk factors—smoking, high blood pressure, low HDL cholesterol, and in most studies, also high total cholesterol—are important predictors of CHD events in elderly as well as middle-aged subjects. Lately, new cardiovascular risk factors such as apolipoproteins, including ApoE, have been a focus of keen investigation **(11).**

Apo E is an essential part of lipoprotein metabolism. It is present in lipoprotein particles and mediates lipoprotein binding to the LDL and lipoprotein remnant receptors. The gene coding for Apo E is located on chromosome 19 near the genes for *Apo* C-I and C-II. Three different alleles, E2, E3, and E4, account for the *Apo E* polymorphism and determine the six genotypes *E2/2, E2/3, E2/4, E3/3, E4/3*, and *E4/4* **(11).**

2.2. The ApoE Gene and its Variants

ApoE is a member of the apolipoprotein gene family, a group of genes that serve a variety of functions related to lipoprotein metabolism*. ApoE* is located at chromosome 19q13.2 and is closely linked to the *ApoC-I/C-II* gene complex. It consists of four exons and three introns spanning 3597 nucleotides. The transcript produces a 299 amino acids polypeptide **(12).**

The genomic organization of *ApoE* is similar to that of the *ApoA* and *ApoC* gene families, suggesting that these genes arose from a common ancestor by gene duplication. The primary product of the *ApoE* gene is a 317-amino acid

protein that gives rise to the 299-amino acid mature protein by cleavage of an 18-amino acid signal peptide **(13).**

The structural gene is polymorphic with three common alleles E2, E3, and E4 that code for three isoforms of the protein, known as E2, E3, and E4. Of these variants, *ApoE3* shows the highest allelic frequency (> 60%) in all populations studied. The variants of the protein of Apo E, E2, and E4, each differ from the wild type E3 by one amino acid. Crude estimates are that *E2* is carried by 3%-20% of a population pool and *E4* by 20%-40%, depending on the racial or ethnic heritage of the population studied **(12).**

2.2.1. Disease burden

The *ApoE* polymorphism has functional effects on lipoprotein metabolism mediated through the hepatic binding, uptake, and catabolism of lipid particles, i.e., chylomicrons, chylomicron remnants, VLDL, and HDL subspecies. ApoE contributes to variability in normal cholesterol levels in populations **(9).**

The major effect of genetic variation at this locus is its influence on cholesterol levels, one of the major risk factors for cardiovascular disease (CVD), particularly coronary artery disease. With reference to cholesterol effects from the E3 allele, E4 is associated with higher total and low density lipoprotein cholesterol levels and E2 with lower levels. The cholesterol lowering effect of E2 tends to be greater than the cholesterol raising effect of E4. In total, the contribution of this gene to cholesterol variability based on a variety of populations that have been studied is no more than 10%. Diet and other genes contribute to each individual's cholesterol level, as well as to the population's average level. Many studies have looked at interactions with variants of this gene as possible modifiers of other cardiovascular risks, such as high- and low-fat diets and active vs. sedentary lifestyles. Interactions with lipid-lowering medications also have been investigated in relation to ApoE **(12).**

2.3. ApoE Genotypes and Plasma Lipoprotein-Lipid Levels

ApoE is a constituent of TG-rich chylomicrons, VLDL particles and their remnants, and a subclass of HDL. The primary role of ApoE in plasma lipid metabolism is to mediate the interaction of chylomicron remnants and IDL particles with lipoprotein receptors, including the LDL receptor and the chylomicron remnant or ApoE receptor. The remnant receptor appears to be the LDL receptor-related protein **(13).**

In populations of European origin, the E3 allele ranges in frequency between 0.7 and 0.8, the E4 between 0.10 and 0.15, and E2 between 0.05 and 0.10. In the general population, the E2 allele is consistently associated with lower levels of total plasma cholesterol, LDL cholesterol, and ApoB and elevated levels of TG and Apo E compared with the *E3* allele. Elevated levels of TG and Apo E are consistent with impaired clearance of remnant particles containing *ApoE2*, presumably due to defective receptor recognition of ApoE2 containing particles. The basis for the reduced Apo B and LDL cholesterol levels in *ApoE2/3* and *2/2* individuals is less clear**. Ehnholm et al (1984**) suggested that the presence of ApoE2 in intestinal VLDL particles impairs their conversion to LDL by interfering with normal lipolytic processing. Conversely, the *E4* allele is associated with higher levels of total and LDL cholesterol and Apo B and lower levels of Apo E. These observations are consistent with the faster rate of catabolism of particles containing *ApoE4* compared with those containing *ApoE3* **(14).**

In general, the lower plasma total cholesterol levels observed in subjects carrying the E2 allele correlate with reduced coronary and peripheral artery atherosclerosis, and the higher cholesterol levels seen in E4 carriers are associated with a higher prevalence of cardiovascular (CV) disease **(13).**

2.4. Studies on *ApoE* Polymorphism

Kataoka et al. (1996) studied *ApoE* polymorphism in American Indians and its relation to plasma lipoproteins and diabetes*. Apo E* was significantly related to glucose control in diabetic women; those with *ApoE* 3 had higher glucose and hemoglobin A1C concentrations. The results of the study showed that (1) American Indians had low frequencies of *ApoE* 2; (2) *ApoE* genotype could influence levels of VLDL, LDL, HDL, Apo B, and Apo A-I; (3) the associations of *ApoE* polymorphisms with lipid parameters differed between men and women; and (4) the associations in women of Apo E polymorphisms with lipid parameters were modified by menopausal status **(15).**

Nduna et al. (1999) investigated the relevance of *ApoE* polymorphism to coronary artery disease in the Saudi population. The polymorphism was determined by PCR - RFLP in 320 Saudi blood donors (BD), 96 CAD patients, and 40 control subjects who had undergone angiography. Compared to controls, only *E4* was elevated in CAD patients. More than 61% ($P < .0001$) of the patients had angina, and 52.1% ($P < .05$) were diabetic; both of these factors were strongly associated with the presence of allele *E2*. The *E2* allele was also associated with hypertension, elevated serum triglycerides, and total cholesterol. On the other hand, the allele *E* 4 appeared to be associated with increased risk of CAD and was also associated with hypertension, vessel disease and restenosis. Accordingly, E4 may be associated with increased risk of CAD, whereas E2 appears to be a predictor of several risk factors for atherosclerosis **(16).**

Evagelos et al. (2004) studied *ApoE* gene polymorphism and its effect on serum lipid parameters in a Greek population originating from Northwestern Greece (n = 555). The allele frequencies were *ApoE*2: 6.3%, *ApoE*3: 80.7%, and *ApoE*4: 13.0%. *ApoE* polymorphism was associated with significant differences in serum lipid, and lipoprotein levels. Particularly, individuals with the *ApoE*2 allele had higher serum T.G and ApoE levels and lower levels of total cholesterol, LDL cholesterol, and ApoB, compared to those with the

alleles *ApoE*3 and *ApoE*4. However, the impact of the *ApoE* 4 allele on lipid parameters seen in other populations was not observed in this population. Furthermore, the combination of *ApoE* polymorphism and serum ApoE concentration explained a larger percentage of serum lipid variability than the polymorphism alone. In conclusion, the results of this study suggested that ethnic differences, as well as alterations of serum apoE levels, significantly modify the relationship between *ApoE* gene polymorphism and serum lipid variability **(17).**

Ariel et al. (1997) tested the hypothesis that ApoE polymorphisms are associated with plasma lipid levels in an elderly, multiethnic population. Cross-sectional data from 1068 institutionalized individuals from Northern Manhattan over the age of 64 who were not on a lipid-lowering diet or drug were analyzed. The ethnic distribution was 34% African-Americans, 47% Hispanics, and 19% non-Hispanic Caucasians. In the entire group, the most prevalent *ApoE* allele was *ApoE*3 (76%), followed by *ApoE*4 (16%) and *ApoE*2 (8%); *ApoE*4 was more prevalent in African-Americans (21%) than in non-Hispanic Caucasians (12%) or Hispanics (14%). The *ApoE*2 allele was the most important correlate of plasma lipids, but this association varied across ethno racial groups. After being adjusted for age, sex, obesity, diabetes mellitus, and alcohol intake, LDL cholesterol levels declined with each *ApoE*2 allele by 8.8 mg/dL in Hispanics and by 25.6 and 18.1 mg/dL in non-Hispanic Caucasians and African-Americans, respectively ($P<.001$). No significant independent effect was noted for any *ApoE* genotype on HDL cholesterol. Overall, there was a reduction in the total/HDL cholesterol ratio, per *ApoE*2 allele, of 0.82 in non-Hispanic Caucasians and 0.43 and 0.48 in African-American and Hispanic individuals, respectively ($P<.05$). In a multivariate model, *ApoE*4 did not significantly affect plasma lipid levels. Plasma triglyceride levels were inversely correlated with the number of *ApoE*4 alleles (175, 159, and 143 mg/dL with 0, 1, and 2 alleles, respectively; $P = .002$), and this effect increased with age. Thus, in an elderly, multiethnic population, *ApoE* polymorphisms were important determinants of blood lipids, with differing effects depending on ethnicity. The presence of

*ApoE*2 was associated with lower LDL cholesterol levels and total/HDL cholesterol ratio, although *ApoE* genotype did not influence HDL cholesterol levels **(18).**

2.5. Studies on *ApoE* gene Polymorphisms and Gene Environment Effects on plasma lipid profile

James et al. (2000) studied *ApoE* gene and gene-environment effects on plasma lipoprotein-lipid levels. Available data generally indicate that *ApoE2*, and possibly E3, genotype individuals reduce plasma total and LDL cholesterol levels more than *ApoE4* individuals with statin therapy. Some evidence also indicates that *ApoE2* individuals are more likely to respond favorably to gemfibrozil and cholestyramine. On the other hand, it appears that with probucol, *ApoE4* genotype individuals may improve plasma lipoprotein-lipid profiles more than *ApoE3* individuals. *ApoE2* and *E3* genotype perimenopausal women appear to improve plasma lipoprotein-lipid profiles more with hormone replacement therapy than *ApoE4* women. On the other hand, low-fat diet interventions tend to reduce plasma LDL cholesterol and, perhaps, plasma total cholesterol levels more in *ApoE4* than in *ApoE2* or *E3* individuals. Both cross-sectional and longitudinal studies generally indicate that *ApoE2* and *E3* individuals improve plasma lipoprotein-lipid profiles more with exercise training than *ApoE4* individuals. Although these data are hardly definitive, they lend strong support for the possibility that in the near future individuals will be directed to what might be their optimal therapy for improving plasma lipoprotein-lipid profiles and cardiovascular disease risk based partially on *ApoE* genotype **(13).**

Weintraub et al. (1987) studied dietary fat clearance in normal subjects as regulated by genetic variation in *ApoE*. They examined the effect of the *ApoE* polymorphism on dietary fat clearance using the vitamin A-fat loading test, which specifically labels intestinally derived lipoproteins with retinyl palmitate (RP). Twenty seven normal subjects were studied, 10 with *E3/3*, 9 with *E3/2*, 7 with *E4/3*, and 1 with *E4/4*. After a vitamin A-containing fatty meal,

postprandial RP concentrations were measured in chylomicron (Sf greater than 1,000) and non chylomicron (Sf less than 1,000) fractions for 14 h. Compared with *E3/3* subjects, E3/2 subjects had a significantly higher nonchylomicron RP concentration ($P < 0.05$) indicating slower clearance and the *E4/3, E4/4* group had a significantly lower nonchylomicron RP concentration ($P < 0.05$) indicating faster clearance. The clearance in the latter group was twice that of *E3/2* subjects ($P < 0.01$). Thus, heterozygosity for the defective form of apo E, E2, delays, and the surprising presence of a functionally normal allele, E4, increases clearance. This ApoE effect on exogenous fat clearance may explain the effect of the *ApoE* genotypes on LDL cholesterol levels **(19).**

Pouliot et al. (1990) investigated the associations between total adiposity, body fat distribution, and plasma lipoprotein levels within groups of women defined on the basis of *ApoE* genotypes, in order to verify whether *ApoE* polymorphism could modify these associations. In women having only *ApoE3* isoforms (n = 24), body fat mass, the waist: hip circumference ratio, and computed tomography-derived total and intra-abdominal fat areas were all positively correlated with VLDL and LDL lipids and ApoB concentrations. These body fatness variables were also negatively correlated with plasma HDL cholesterol concentration. These associations were, however, altered in the groups of women carrying either *ApoE2* or *E4* isoforms. Indeed, in women carrying the *ApoE2* isoform (n = 22), body fatness variables were predominantly associated with significantly greater VLDL components concentration and with LDL triglyceride content. No association was found between adiposity and LDL cholesterol or Apo B levels in these women. In contrast, no relationship was found between total adiposity, regional fat accumulation, and VLDL fraction in women carrying the *ApoE4* isoform (n = 17). In this latter group, computed tomography-measured total abdominal fat accumulation was positively correlated with LDL Apo B concentration, whereas

intra-abdominal fat accumulation was positively correlated with both LDL cholesterol and Apo B concentrations **(20).**

Raiha et al. (1997) tested the effect of serum lipids, lipoprotein fractions, and *ApoA-1, B*, and *E* on mortality from vascular and nonvascular causes in an unselected elderly population. The random sample of 347 community-living individuals aged 65 years or older was obtained in 1982. Serum total cholesterol, LDL, HDL, triglyceride, and Apo A-1, B, and E were determined at baseline. After the 11-year follow-up, 199 of the participants had died, and 148 were still alive. Mortality data from vascular and nonvascular causes by the end of 1993 were obtained from official registers. In the univariate analysis, a low total cholesterol level was associated with death due to both vascular and nonvascular causes. After the adjustment for other risk factors, the inverse association between total cholesterol and vascular mortality disappeared, but low total cholesterol was still a significant predictor of death due to nonvascular causes. Adjusted relative risks (RRs) of death due to nonvascular causes for those with elevated total cholesterol (5.1 to 6.5, 6.6 to 8.0, and >8.0 mmol/L) compared with the reference group (≤5.0 mmol/L) were 0.5 (95% confidence interval [CI], 0.2 to 1.2), 0.6 (0.2 to 1.0), and 0.2 (0 to 0.8), respectively. Neither concentrations of HDL, LDL, triglyceride, nor *ApoB* were associated with vascular or nonvascular mortality. On the other hand, low concentration of Apo A-1 predicted vascular death. The RR for the lowest tertile was 1.6 (1.1 to 2.5) compared with the highest tertile. Furthermore, the occurrence of the *ApoE4* allele was associated with increased risk of vascular mortality (RR, 1.5; 95% CI, 1.0 to 2.2), but the risk was not related to the levels of lipids, lipoproteins, or other apolipoproteins at baseline. Nonvascular mortality also tended to be predicted by the presence of the *E4* allele (RR, 1.5; 95% CI, 0.9 to 2.5). In an unselected elderly population, the allelic variation of *ApoE*, i.e. the presence of the *E4* allele, and a low concentration of apo A-1 were more accurate indicators of vascular mortality than total cholesterol or lipoprotein fractions.

The risk associated with the *ApoE* polymorphism was however unrelated to dyslipidemia **(21).**

Yang et al. (2001) investigated the relationship between *ApoE* polymorphism and the early onset of (CHD) and the effect of apoE on lipids and lipoproteins in healthy Chinese subjects. Eighty six patients with CHD younger than 55 years (CHD1), 136 patients with CHD older than 65 years (CHD2), and 136 healthy subjects were enrolled, and their plasma levels of TG, total cholesterol (TC) and HDL were determined. The *ApoE* genotypes were identified by PCR-RFLP. According to the study *ApoE 3/4* genotype and *E4* allele frequency in the CHD1 group were higher than those in the CHD2 group and healthy subjects, while no differences were found between CHD2 and healthy subjects. Meanwhile, the plasma levels of TC and LDL were higher in the CHD2 group than in both CHD1 group and healthy subjects. Each apoE isoprotein has variable TC and LDL-C levels that is E2 (E2/2 + E2/3) < E3 (E3/3) < E4 (E4/4 + E3/4). The authors concluded that ApoE2 is one of the genetic factors that affect TC and LDL-C levels, and *ApoE4* has a very close relation to CHD, suggesting that *ApoE4* is an independent genetic factor of the early onset of CHD **(22).**

Zaman et al. (1997) studied the association of apolipoprotein genetic polymorphisms with plasma cholesterol in a Japanese rural population. They measured plasma TC and HDL in a cross-sectional sample of 1328 (462 men and 866 women) Japanese (aged 40 to 80 years).They found that the polymorphism of the *ApoE* gene is significantly related with TC and the TC:HDL-C ratio. The *ApoE2* carriers have lower levels of TC and a lower TC: HDL-C ratio, *ApoE 3* carriers have intermediate levels, and *ApoE4* carriers have higher levels. These findings held whether sexes were analyzed separately or together. Although an opposite trend in HDL-C levels was observed, it did not reach the level of statistical significance. Multiple regression analyses have shown that *ApoE* polymorphism accounts for about 2.3% of the variation in TC and TC: HDL-C ratio irrespective of sex. When the

subjects were divided into two groups according to their age (< or = 59 and > or = 60 years old), the effect of ApoE alleles on serum cholesterols appeared to be diluted in the younger age group whereas it appears to be accentuated in the older group for both sexes. These findings suggest that among the apolipoprotien genetic polymorphisms examined, the *ApoE* gene may be considered as a possible candidate for the "high-risk" strategy of atherosclerosis prevention in the Japanese population **(23).**

Sheehan et al. (2000) examined the relationship between serum cholesterols and *ApoE* genotypes in a cohort of healthy Irish adults. One hundred healthy Irish adults, aged 19-65 years, were recruited from the Cork City area. While the *E2* (12%) was the least prevalent, *E3* was the most prevalent *ApoE* genotype (66%) in this group of healthy Irish adults. A significant *ApoE* gene-dosage effect was evident, whereby individuals with the *ApoE2* genotype had a lower level of total cholesterol, *E3* had intermediate levels, and *E4* had a higher level. Moreover, those with the *ApoE4* genotype had a significantly higher level of LDL-C compared to *E2* or *E3* genotypes. There was no significant difference in mean serum adjusted HDL-C levels between the three *ApoE* genotypes. The findings of this study suggest that healthy Irish adults with the *ApoE4* genotype have higher serum total and LDL-C levels than those with *E2* or *E3 ApoE* genotypes and therefore may have a higher risk of atherosclerotic coronary artery disease and coronary heart disease in later life **(24).**

Cubrilo et al. (1998) investigated the metabolic risk markers according to various *ApoE* genotypes, in order to evaluate a possible risk for CHD. Their results revealed that the frequencies of *ApoE3/3* are the most frequent (46%), followed by *E4/4* (2%), *E3/4* (14%), *E2/3* (14%), and *E2/4* (2%) in the middle-aged women. Higher mean TG, LDL-C and apo B levels were found with *ApoE3/4,* and lower mean levels of HDL-C. Serum lipoprotein Lp (a) concentration was higher in women with genotypes *E3/3*. ApoE concentration

was the lowest with genotypes E4/4, i.e., the highest with *E2/3*. Serum TC tended to be higher in women with genotypes *E4/4.* Genotype *E3/4* was connected with the highest concentrations of triglycerides, LDL, Apo B and with the lowest concentrations of HDL in the relation to the other analyzed genotypes. This group of women could possibly represent high risk women for CHD. Genotype *E3/3* was associated with the highest concentration of independent genetic risk marker for CHD. The genotype *E4/4* has the highest concentration of TC, and has to be taken in account for risk evaluation in women. High level of ApoE and low level of apo A-I were associated with *E2/3* genotypes. The significance of *E3/4* with the high total/HDL-C ratio was an important indicator, because total/HDL-C ratio represents independent established risk factor (ERF) for CHD. The authors concluded that *ApoE* genotypes as genetic markers and investigation of serum metabolic risk markers appear to be important in view for further evaluation of high risk women for CHD in the studied population **(25).**

Ranjith et al. (2004) examined the association of the Lp(a) promoter pentanucleotide repeat polymorphism and the *ApoE* codon 112 and 158 genotypes in 195 young South African Indian patients (< or = 45 years) with myocardial infarction (MI). Their study results were compared with 300 healthy age-matched control subjects drawn from the same community and 107 unaffected siblings (18-45 years). In addition, fasting lipograms were performed on all patients and a detailed history of conventional risk factors and family background was obtained. Of the six different Lp (a) alleles detected, the 8-repeat sequence was most frequently seen. However, no difference in frequencies existed between patient and control groups. The most frequently occurring *ApoE* genotype in the three study groups was *E3/E3* (patients 71%; siblings 70%; controls 70%). A significant difference in the *E3/E4* genotype was seen between patients and controls (23% vs 14%) and between siblings and controls (24% vs 14%) These patients were also more likely to have significantly higher LDL and lower HDL levels. No association was observed

between any of the Lp(a) or *ApoE* genotypes and conventional risk factors such as smoking, diabetes, hypertension, obesity or a family history of CHD. In conclusion, the *ApoE3/E4* genotype is strongly associated with the incidence of MI in young South African Indians. This genotype also adversely affects LDL and HDL levels, both of which contribute to premature atherosclerosis. In contrast, the Lp(a) pentanucleotide repeat polymorphism does not appear to have any etiological role in MI in this population **(26).**

Shin et al. (2005) determined the effects of polymorphisms in the *Apo E* gene on lipid levels in Korean adults and investigated the interactions between these polymorphisms and environmental factors in determining lipid levels. The investigators performed a cross-sectional study of 1,900 subjects (668 men and 1,232 women; 45-74 yr old) in Namwon, Korea, in 2004. *ApoE* polymorphisms were determined by PCR - RFLP analysis. Carriers of the *ApoE2* allele had significantly lower TG and LDL-C concentrations than did carriers of the *ApoE3* or *ApoE4* alleles, regardless of gender. The *ApoE* allele type had significant effect on HDL-C and TG levels in women, but not in men. The effect of *ApoE* allele type on HDL-C levels was modified by age in women. In addition, in men, the effect of *ApoE* allele type on TG levels was modified by smoking. These findings highlight the important effect of gene-environment interactions on lipid levels **(27).**

Kim et al. (1993) studied *ApoE* genotypes of normal and hyperlipidemic subjects to find out the frequency of ApoE isoform distribution in the Korean population. The *ApoE* allele frequency in 73 normal subjects was 4.8% for *E2*, 84.9% for *E3* and 10.3% for *E4*. In diabetic patients with hyperlipoproteinemia, the frequency of Apo E allele was 6.3% for E2, 81.0% for E3 and 12.7% for E4. There was no significant difference in apo E isoform distribution between diabetics and normal populations. But in patients with cardiovascular disease with hyperlipidemia, the *ApoE4* allele frequency was significantly higher than normal (20.0% vs 10.3%). *ApoE3* was the most common isoform in normal and diabetic subjects and apo E2 isoform was rather of lower frequency compared to Caucasians. This pattern is similar to the

Japanese population but somewhat different from other populations. From the data of a high association of ApoE4 allele and cardiovascular disease with hypercholesterolemia, ApoE isoforms may be one of the determinants of hyperlipoproteinemia **(28).**

Scaglione et al. (1999) studied the role of lipid, apolipoprotein levels and *ApoE* genotype in young Italian patients with MI. In this study, the conventional risk factors, lipids and apolipoproteins, and *ApoE* allele distribution were evaluated in 98 consecutive acute MI survivors (94 males, 4 females) aged 40.1 +/- 3.9 for at least three months after their acute event. These survivors were matched for age, sex, body mass index and presence of diabetes mellitus with 98 controls selected from subjects admitted to the same hospital for other reasons. Coronary angiography during hospitalization showed the absence of critical stenosis in 6.6% of the survivors, mono-vessel disease in 57.7%, and multi-vessel disease in 35.5%. The survivors had a higher frequency of smoking, hypertension, family history for CAD and dyslipidemia, and a much greater frequency of 3 or more risk factors than the controls. No differences were observed in *ApoE* allele distribution (*ApoE4* 0.11 vs 0.08, *ApoE3* 0.86 vs 0.89, *ApoE2* 0.03 vs 0.03), nor in lipid profile when both higher risk genotype (*E3/4, E4/4, E2/4*) and lower risk genotype groups (*E2/2, E2/3, E3/3*) were analyzed. Odd ratios (ORs) were calculated as measures of the association of the *E4*-positive genotypes with AMI. They indicated a non-significant increase in risk of AMI when the survivors were compared with the controls (OR 1.78, 95% CI 0.84-3.70, p = 0.13). This study provides further evidence that conventional coronary risk factors are usually present in young AMI patients. The *ApoE4* allele was associated with a 1.8 non-significant increase in the risk of AMI in subjects group with premature CAD. Comparison with controls showed that the presence of three or more risk factors sharply increased the probability of premature CAD and that hyper-triglyceridemia is an independent risk factor **(29).**

Lehtimaki et al. (1995) studied the association between serum lipids and *ApoE* genotype as influenced by diet in a population-based sample of free-living children and young adults. One thousand and twelve subjects, aged 9-24 years, were studied as a part of the Cardiovascular Risk in Young Finns Study in 1986. Serum lipid concentrations and apoE phenotypes were determined, and the composition of the diet was assessed by the 48-h recall method. The subjects were divided into three groups according to the intake of dietary saturated fatty acids (SAFA, g/1000 kcal) and cholesterol (mg/1000 kcal). Group one (high SAFA-cholesterol group) was formed from subjects belonging to the highest tertiles of both cholesterol and SAFA intakes (n = 175); group two (middle SAFA-cholesterol group) consisted of subjects belonging to the middle respective tertiles (n = 119); and group three (low SAFA-cholesterol group) consisted of subjects belonging to the lowest respective tertiles (n = 192). The statistical significance of the association of serum TC and LDL concentration with ApoE phenotype increased from the low SAFA-cholesterol group (P = 0.024 for TC and P = 0.015 for LDL-C, respectively) to the high SAFA-cholesterol group (P = 0.0022 and P = 0.00073, respectively). The middle SAFA-cholesterol group fell between these two groups **(30).**

Gonzalez et al. (2000) studied the effect of genetic variations of the *ApoE* gene on the plasma triglyceride levels after heart transplantation. A cohort of 103 recipients of heart transplant (93 men and 10 women, with a mean age of 47 +/- 13 years) under triple immunosuppressive therapy were submitted to a genetic study of the *ApoE* gene region. Anthropometric and analytical data, including lipid profile and arterial blood pressure were collected prior to transplantation and 3, 6, 12, and 24 months after it. Sixty five subjects had the genotype E3/E3, 27 the genotype *E3/E4*, 6 the genotype *E2/E3*, and 5 the genotype *E2/E4*. Carriers of the *E2* allele (that is, genotypes *E3/E2* and *E4/E2*) had higher plasma TG levels after 3 months (3.47 +/- 1.88 mmol/liter $p < 0.001$) and after 1 year of transplantation (3.13 +/- 1.77 mmol/liter $p < 0.05$) than the other genotypes. There were no differences, however in the plasma levels of TC, LDL-C, and HDL-C. The authors concluded

that the presence of the *E2* allele in heart-transplant recipients produced a greater rise in total TG plasma levels than the other genotypes **(31).**

Lin et al. (2004) studied association of *Apo E* genotypes with serum lipid profiles in a healthy population of Taiwan. In the study population, the frequency of *ApoE* allele E3 was greatest (85.2%); the frequency of E2 was 8.4%; and that of E4 was 6.4%. The serum apoA-1/apoB ratio showed significant difference among the 3 apoE genotype groups (p 0.0001); the apoA-1/apoB ratio was 1.9 +/- 0.1 (mean +/- SD) in the E2 group, vs 1.4 +/- 0.04 and 1.5 +/- 0.12 in the E3 and E4 groups, respectively. No significant associations were found between *ApoE* alleles and the serum levels of the various lipids or other CHD risk factors **(32).**

CHAPTER 3

Materials and Methods

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Materials and Methods

3.1. Materials

3.1.1. PCR primers

Oligonucleotide primers for the PCR were designed from published nucleotide sequences (Table 3.1).

Table 3.1. PCR primers sequence

Gene	Sequence 5́ to 3́	Annealing temp	Reference
ApoE	TCCAAGGAGCTGCAGGCGGCGCA	76^0C	**(33)**
ApoE	GCCCCGGCCTGGTACACTGCCA	77^0C	**(33)**

F: forward, R reverse, (All primers were synthesized by Operon Biotechnologies, Germany).

3.1.2 Kits

- DNA extraction kit (Promaga, USA)
- Triglyceride kit (DiaSys**,** Germany)
- Cholesterol kit (DiaSys**,** Germany)

3.1.3. Reagents and Chemicals

- HDL (DiaSys**,** Germany)
- Normal Cholesterol control (DiaSys **,** Germany)
- High Cholesterol control (DiaSys **,** Germany)
- Normal Triglyceride control (DiaSys **,** Germany)
- High Triglyceride control (DiaSys, Germany)
- Agarose Molecular biology grade (Promaga, USA)
- DNA molecular weight marker 50, 100bp (Promaga, USA)
- EDTA disodium salt (Promaga, USA)
- Absolute Ethanol (Sigma, USA)
- Ethidium bromide (Promaga, USA)
- Absolute Isopropanol (Sigma, USA)
- Tris base " hydroxyl methyl amino methane" (Promaga, USA)
- DNAse, RNAse free water (Promaga, USA)

- Dimethyl sulfoxide (DMSO)
- Restriction enzyme buffers
- BSA
- Orange G

3.1.4. Enzymes

- Restriction enzyme HaeII
- Restriction enzyme AflIII

3.1.5. Apparatus and Equipments

- Thermal Cycler (Eppendrof Mastercycler, personal)
- L.G. Microwae Oven
- Spectrophotometer UV-Vis
- Water Bath
- Electrophoresis Apparatus
- Vortex Mixer
- Digital Camera
- Power Supply (Biorad)
- Freezer, Refrigerator
- Micro-Centrifuge
- Hoefer shortwave UV light table (Transilluminator)
- Computer
- Electrical Balance

3.2. Methods

3.2.1. Study population

This study is a retrospective case- control study in which *ApoE* genotyping and lipid profiling were performed on 137 individuals who were randomly selected. Sixty nine subjects (24 female and 45 male) with coronary artery disease from Nasser hospital and sixty eight normal subjects were included in this study (35 female and 33 male). All subjects were asked to fill a questionnaire, a sample of which is presented in Appendix B.

3.2.2. Sample collection

Blood samples were collected from the patients and the control subjects after a 12- to 16-hour overnight fasting.

Each sample was collected into two tubes one EDTA tube and the other was placed in a glass tube, the sample in the glass tube was centrifuged at 2500rpm for 5 minute and separated serum was used for lipid profiling by the use of commercial kits. The EDTA sample kept at 4 ^{0}C was used within 24 hours for DNA extraction and subsequent PCR analysis.

3.2.3. Ethical Considerations

An authorization to carry out the study was obtained from Helsinki (Declaration of Helsinki the most widely accepted guideline on medical research involving human subjects) using an agreement letter prepared by the Islamic University of Gaza.

Subjects gave their consent to participate in the study. All the information that were obtained about the subjects was kept confidential (refer to Appendix C for more details).

3.2.4. Data Analysis

The data were entered, stored and analyzed by a personal computer using SPSS 8.0 statistical package. Differences in proportions were assessed by ANOVA, t- test, Chi square test, P values < 0.05 were considered statistically significant.

3.3. Lipid Profiling

3.3.1. Serum cholesterol determination

Sample /standard was mixed with reagent in the proportions shown in Table 3.2. below.

Table 3.2. Sample /standard preparation for cholesterol determination

	Blank	Sample or standard
Sample or standard	-	10µl
Distilled Water	10µl	-
Reagent	1000 µl	1000 µl

The contents of each tube were mixed and incubated for 20 min at 20-25^{0}C then the absorbance of each tube was read at wave length 500nm against the blank.

Cholesterol content was then calculated by applying the following equation

Cholesterol [mg/dl] = $\frac{\text{Sample absorbance X Standard Concentration}}{\text{Standard absorbance}}$ **(34,35)**

3.3.2. Serum triglyceride determination

Sample /standard was mixed with reagent in the proportions shown in Table 3.3. below.

Table 3.3. Sample /standard preparation for triglyceride determination

	Blank	Sample or standard
Sample or standard	-	10µl
Dist. Water	10µl	-
Reagent	1000 µl	1000 µl

The contents of each tube were mixed and incubated for 20 min at 20-25^{0}C then the absorbance of each tube was read at wave length 500nm against the blank.

Triglyceride content was then calculated by applying the following equation

Triglyceride [mg/dl] = $\frac{\text{Sample absorbance X Standard Concentration}}{\text{Standard absorbance}}$ (**34,35)**

3.3.3. HDL-C measurement

HDL-cholesterol was measured after chylomicrons; VLDL and LDL were precipitated by adding phosphotungestic acid to the sample. Centrifugation afterwards leaves only the HDL in the supernatant. The HDL-C content was determined enzymatically using Ecoline S + cholesterol as described below.

Assay procedure

200 µl serum samples were mixed with 500 µl precipitation reagent (phosphotungestic acid and magnesium chloride) and the mixture was incubated for 15 min at room temperature, then the mixture was centrifuged for 20 min at speed of 2500 rpm., 0.1ml of the clear supernatant was then transferred to the reaction solution for determination of cholesterol

The supernatant after the centrifugation should be clear, serum or plasma with triglyceride contents ≥1000 mg/dl tends to produce turbid supernatant or floating precipitates, in this case the sample should be diluted 1 :1 with NaCl solution (0.9%) and then precipitation should be performed and the final result should be multiplied by 2.

3.3.4. LDL calculation

LDL can be calculated from the parameters determined above using the following formula:

LDL = total cholesterol - [HDL + $\frac{\text{triglyceride}}{\mathbf{5}}$] **(34,35)**.

3.4. *ApoE* Genotyping

3.4.1. DNA extraction

DNA was isolated from fresh EDTA whole blood cells by using Promaga kit for human DNA isolation

The kit contains the following components that are enough for purifying genomic DNA from 100 samples of human blood:

- Nuclei lysis solution
- Cell lysis solution
- RNase solution
- Protein precipitation
- DNA rehydration solution

The human genomic DNA was isolated from human blood sample according to the kit instructions and was as follows

Three hundred µl EDTA blood were transferred to sterile 1.5 ml micro-centrifuge tube containing 900 µl of cell lysis solution. The tube was inverted 5-6 times to mix the components.

The mixture was incubated for 10 minutes at room temperature (with gentle mixing once during the incubation) to lyse the red blood cells. The tube was then centrifuged at 13,000 rpm for 20 seconds at room temperature.

Supernatant was removed and discarded as much as possible without disturbing the visible white pellet. Approximately 10-20µl of residual liquid should be left in tube.

The tube was vortexed vigorously until the white blood cells were completely resuspended.

Three hundred µl nuclei lysis solution was then added to the tube containing the resuspended cells and the suspension was mixed by pipetting the solution 5-6 times to lyse the white blood cells. The solution should become very viscous.

RNase solution (1.5 µl) was added to the nuclear lysate and mixed with the sample by inverting the tube 2-5 times. The mixture was incubated at 37 ^{0}C for 15 minutes, and then cooled to room temperature.

100µl of protein precipitation solution was added to the nuclear lysate and the mixture was vortexed vigorously for 10-20 seconds. Small protein clumps may be visible after vortexing, and were removed by centrifuge precipitation at 13,000 rpm for 3 minutes at room temperature.

The supernatant was transferred to a clean 1.5 ml micro-centrifuge tube containing 300µl isopropanol at room temperature. The mixture was gently

mixed by inversion until the white thread-like strands of DNA form a visible mass. The DNA was then precipitated by centrifugation 13,000 rpm for 1 minute at room temperature. The DNA would be visible as a small white pellet.

The supernatant was decanted and one volume of 70% ethanol kept at room temperature was added to the DNA the tube and gently inverted several times to wash the DNA pellet and the sides of micro centrifuge tube. After centrifugation at 13,000 rpm for 1 min, the ethanol was aspirated using a suitable pipette. The DNA pellet is very loose at this point and care must be used to avoid aspirating the pellet into pipette. The tube was inverted on clean absorbent paper for 10-15 minutes in order to air-dry the pellet.

DNA rehydration solution was added to the dry pellet and the DNA was rehydrated by incubation at 65^{0}C for 1 hour. Periodically the solution was mixed by gently tapping the tube. The DNA was then stored at 2-8^{0}C.

3.4.2. Detection and measurement of extracted DNA

3.4.2.1. Agarose gel electrophoresis

The quality of the isolated DNA was determined by running 5 µl of each sample on ethidium bromide stained 1.0% agarose gels and the DNA was visualized on a short wave U.V. transilluminator, the results were documented by photography.

3.4.2.2. Spectrophotometry

The optical density (O.D) at 260nm of a diluted fraction of the isolated DNA sample was measured by a spectrophotometer and the DNA concentration was calculated by considering 1 $O.D._{(260\ nm)}$ = 50ug/ml DNA and taking into account the dilution factor.

3.5. PCR amplification of *ApoE* gene

3 µl (~150ng) of prepared DNA template was added to 25 µl master mix and 2.5 µl of DMSO (5%) in 0.2 ml thin walled microfuge tube. The volumes and concentrations of a typical PCR reaction are shown in Table 3.4.

Microfuge tubes were then placed in a thermal cycler and PCR amplification was done according to the program described below.

Upon completion of PCR, the products were analyzed by electrophoresis on 2% ethidium bromide stained agarose gel, or the 218 bp amplicons were stored at 4^0C until analysis and digestion.

Table 3.4. Composition of PCR master mix.

Reagent	Composition
dNTPs	400µM each: dATP, dGTP. dCTP, dTTP
Taq DNA Polymerase	50 units/ml
$MgCl_2$	3mM

Table 3.5. PCR reactions.

Reagent	PCR reaction mixture		Final Con. /test
	Initial concentration	Volume	
Master mix		12.5µl	
Primer1	100µM	0.5µl	2.0µM
Primer2	100µM	0.5µl	2.0µM
Template DNA	~200ng	3.0µl	
Nuclease free water		7.25µl	
DMSO		1.25µl	5%

Sequence of the primers is given in Table 3.1.

3.5.1. Temperature cycling program

The thermal cycler program was set as follows:

Step1. Denaturation for 3 minutes at 95 ^{0}C

Step 2. 40 cycles of:

2.1. Melting for 60 seconds at 95 ^{0}C

2.2. Annealing for 60 seconds at 58 ^{0}C

2.3. Extension for 90 seconds at 72 ^{0}C

Step3. Final elongation for 10 minutes at 72 ^{0}C

3.5.2. Expected PCR results

The amplicon (PCR product) generated from *ApoE* gene should yield a 218 bp long ds. DNA fragment. A negative control (with water instead of the DNA template) was included in each reaction. The size of the amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA) run on the same gel.

3.5.3. RFLP analysis

Genotyping for *ApoE* was performed by digesting the PCR product with restriction enzymes (HaeII and AflIII) and separation of the resulting DNA fragments on 3% agarose gels.

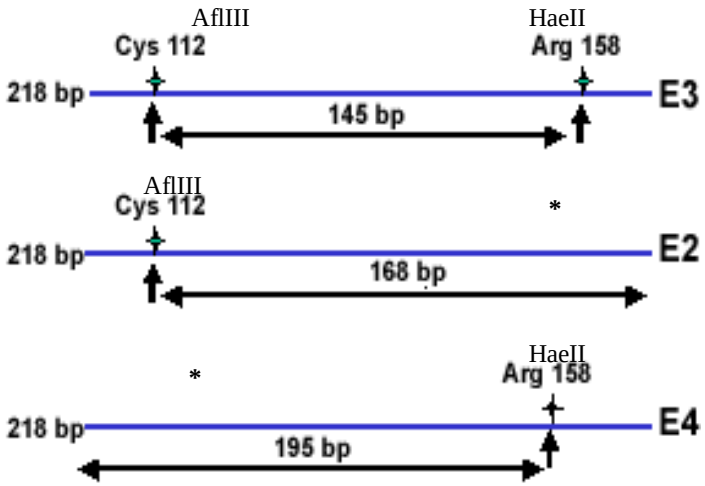

Figure 3.1. A Schematic diagram illustrating *ApoE* RFLP analysis

For RFLP analysis the following mixtures were prepared in clean two 0.2ml microfuge tubes.

Table 3.6. RFLP reaction

Reagent	Volume in tube A (µl)	Volume in tube H (µl)
PCR product	10	10
10X buffer	2	2
AflIII (5.000U/ml)	0.4	-
HaeII (20.000U/ml)	-	0.25
BSA	0.2	0.2
H_2O	7.4	7.55
Total	**20 µl**	**20 µl**

The contents were incubated for 24hours at 37 ^{0}C. The digests were resolved on 3% ethidium bromide stained agarose gels and the results were documented by photography.

CHAPTER 4
RESULTS

CHAPTER 4
RESULTS

4.1. Study Population

The study population consisted of 137 subjects (69 cases, 68 controls). The mean age of subjects was 50.88 ± 13.66. The percentage of males was 56.9% while that of females was 43.1% (Figure 4.1). The distribution of subjects according to their residency was as follows: 28.5% from Rafah, 46.7% from Khan-Younis, and 24.8% from Gaza city (Figure 4.2). About one fifth of the subjects were smokers, while about 81% were non-smokers (Figure 4.3). Most of the study population was non-hypertensive subjects (86.1%), while 13.9% of them were hypertensive (Figure 4.4). Mostly, the study population was non-diabetic subjects (98.5%). Most of the subjects had no family history of coronary heart diseases.

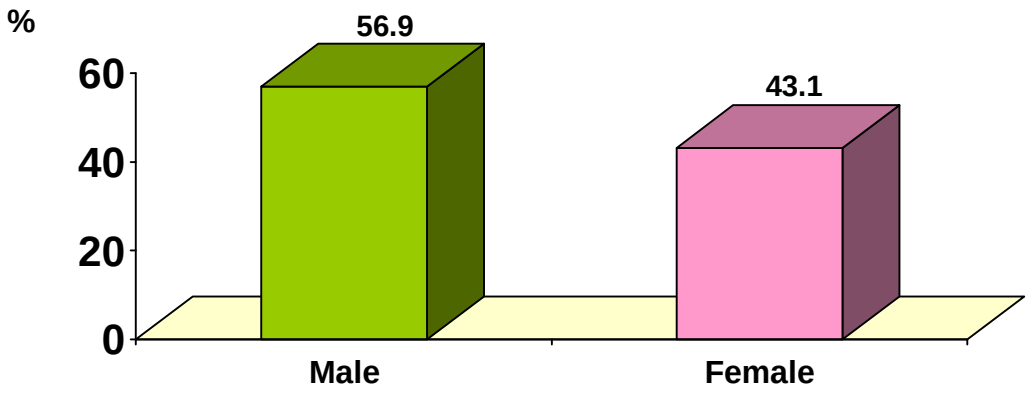

Figure 4.1. Distribution of the subjects according to the gender

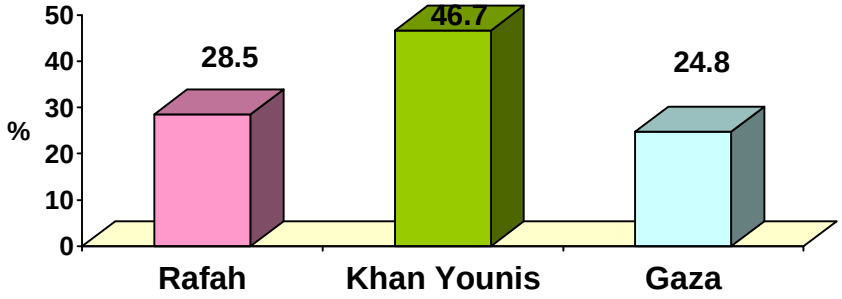

Figure 4.2. Distribution of the subjects according to residency

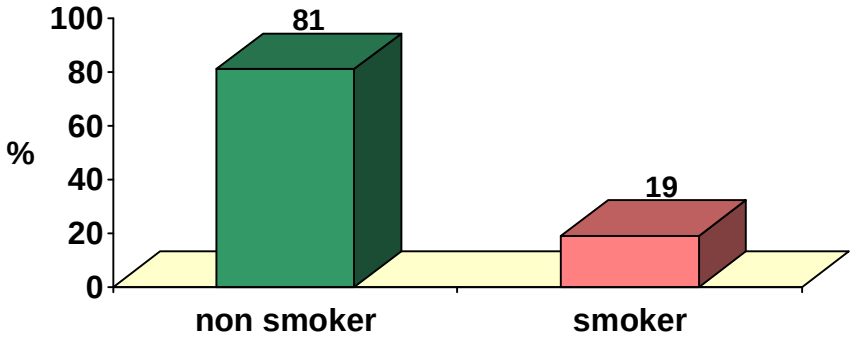

Figure 4.3. Distribution of the subjects according to smoking

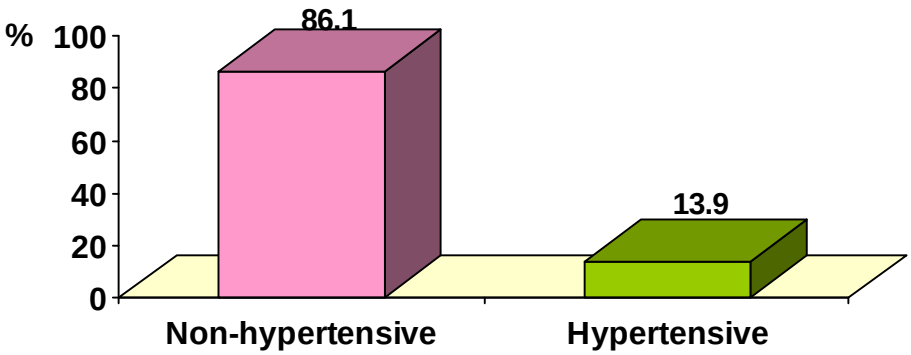

Figure 4.4. Distribution of the subjects according to hypretension

4.2. Relationship between CHD and Demographical Variables

4.2.1. CHD and gender

The results revealed that there is a statistically significant correlation between CHD and gender of the subjects (X^2 = 3.9, p-value= 0.049), where most of cases (65.2%) were males and more than half of the controls (51.5%) were females (Table 4.1).

Table 4.1. Relationship between CHD and gender.

Gender	Case group		Control group		Total		p-value
	n	%	n	%	n	%	
Male	45	65.2	33	48.5	78	56.9	0.049
Female	24	34.8	35	51.5	59	43.1	
Total	69	100	68	100	137	100	

4.2.2. CHD and age

The results of the study showed that there is a statistically significant difference between the means of age of cases and controls (p-value < 0.05) where the mean age of cases was 57.65 years, while that of the controls was 44.01.

4.2.3. CHD and smoking

The results of the study showed that there is a statistically significant difference between the smokers and non-smokers in cases and controls (X^2= 4.56, p-value= 0.049) as shown in Table 4.2.

Table 4.2. Relationship between CHD and smokers.

Subject	Smokers						
	Smokers		Non-		Total		p-value
	n	%	n	%	n	%	
CHD	18	69.2	51	45.9	69	50.4	0.049
Control	8	30.8	60	54.1	68	49.6	
Total	26	100	111	100	137	100	

4.3. PCR Results

4.3.1. Quality of the isolated DNA

Regarding the method of DNA isolation that was described in chapter 3, the quality and quantity of DNA were suitable for PCR processing. The following Figure (4.5) represents the quality of the isolated DNA from human blood samples.

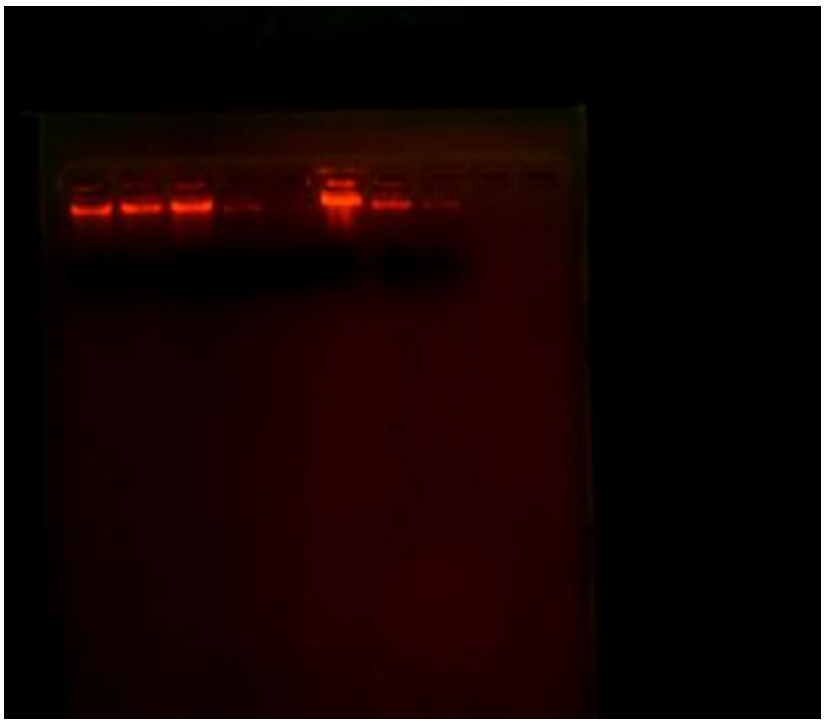

Figure 4.5. A representative photograph of genomic DNA isolated from human blood. The samples were run on ethidium bromide stained 1% agarose gel.

4.3.2. PCR amplification product

A representive photograph of *ApoE* PCR amplification product is illustrated in Figure (4.6) below. Lane 1 in the figure shows the 100bp ladder, lane 4 contains a negative control and the other lanes show the 218 bp *ApoE* amplicon.

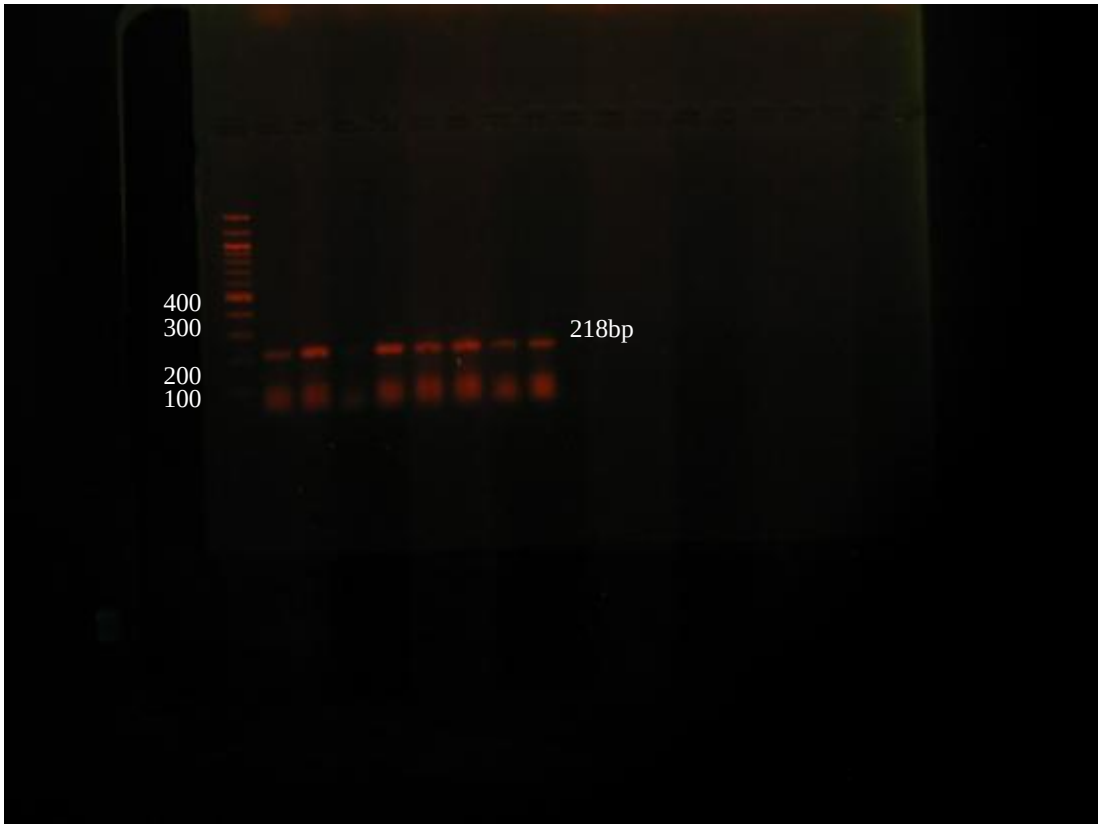

Figure 4.6. A photograph of *ApoE* amplification product. Lane 1: 100bp ladder, lane 4 negative control and the rest of lanes show the 218 bp *ApoE* PCR product.

4.3.3. RFLP result

Figure 4.7. Illustrates a representative *ApoE* genotyping by AflIII (A) and HaeII (H) digestion of the 218-bp amplified fragment.

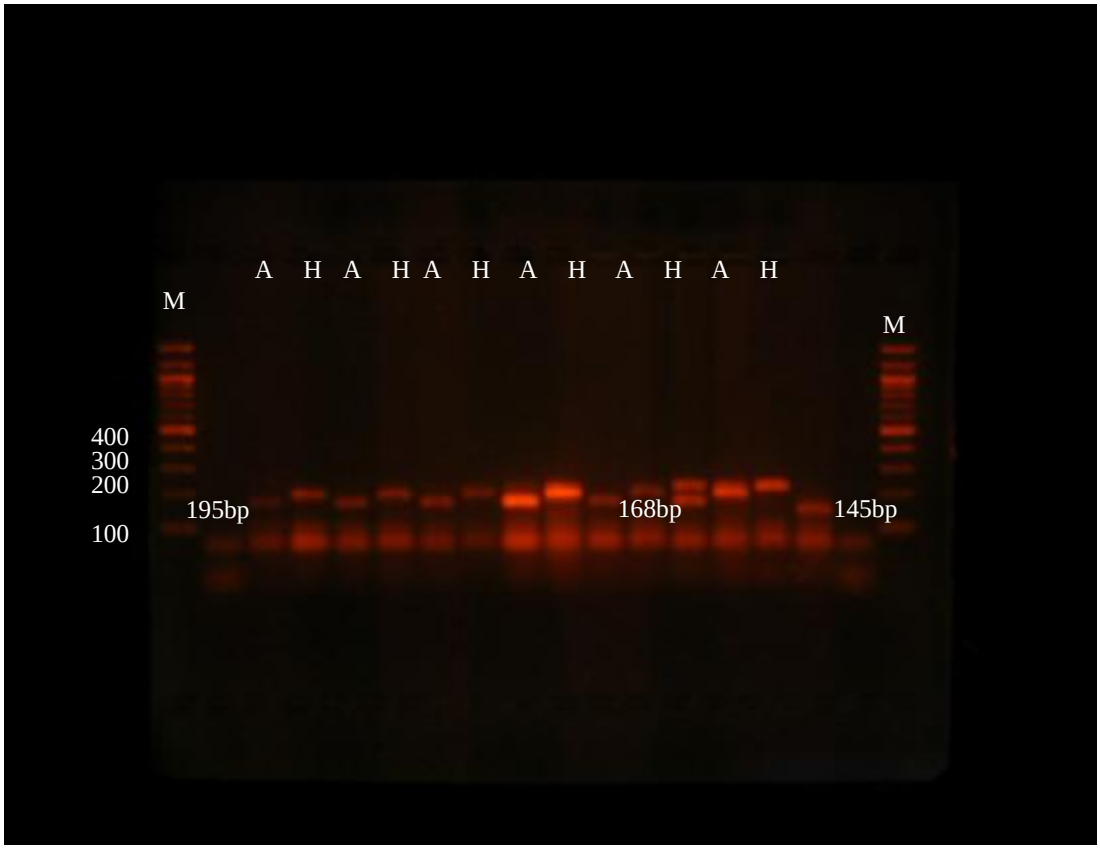

Figure 4.7. A representative photograph of AflIII (A) and HaeII (H) digestion of the *ApoE* 218-bp amplified fragment. M indicates the 100 bp size marker. The digests were run on ethidium bromide stained 3% agarose gel ,E3 =145bp, E2 =168bp, E4=195bp.

4.4. Genotype Frequencies

The *ApoE* allele frequencies in the control subjects were 5.15% for the E2 allele, 87.5% for the E3 allele and 7.3% for the E4 allele. The frequencies in the CHD group were 3.62% for the E2 allele, 82.6% for the E3 allele and 13.77% for the E4 allele (Table 4.3).

Table 4.3. Frequency of ApoE alleles among the case and the control groups

Alleles	CHD group		Control group	
	n	%	n	%
E3	114	82.60	119	87.5
E2	5	3.62	7	5.15
E4	19	13.77	10	7.35
Total	138	100	136	100

4.5. Distribution of Subjects according to *ApoE* Genotypes

The distribution of the subjects according to ApoE genotypes were as follows, 70.1% E3/E3, 21.2% E3/E4, and 8.8% E3/E2 (Figure 4.8).

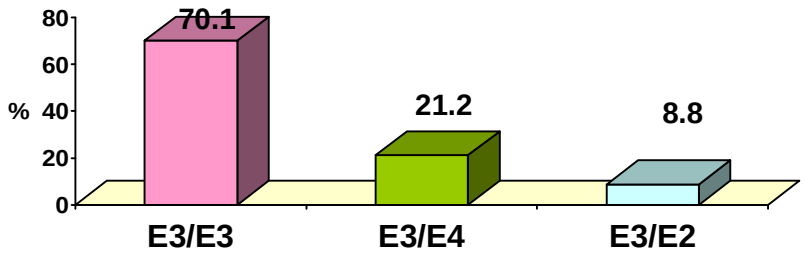

Figure 4.8. Disttribution of the subjects according to ApoE genotypes

4.6. Relationship Between CHD and *ApoE* gene Polymorphism

The results showed that there was no statistically significant relation between CHD and *ApoE* gene polymorphism (X^2 = 3.4, p-value= 0.17), where the presence of E3/E3 genotype was the most frequent among cases and controls (65.2%, 75%, respectively) as shown in Table 4.4.

Table 4.4. Distribution of the *ApoE* genotypes among the cases and the control groups

Genotype	Case		Control		Total		p-value
	n	%	n	%	n	%	
E3/E3	45	65.2	51	75	96	70.1	0.17
E3/E2	5	7.2	7	10.3	12	8.8	
E3/E4	19	27.5	10	14.7	29	21.2	
Total	69	100	68	100	137	100	

4.7. Relationship Between CHD and Lipid Profile

The data indicated in Table 4.5 show that there is no statistically significant difference between the means of cholesterol and triglyceride levels between the CHD and the control groups (t= 1.3, p-value= 0.19), (t= 1.6, p-value= 0.1), respectively. On the other hand there was significant differences between the mean of LDL and HDL levels between cases and controls (p-value= 0.02 and < 0.05), respectively.

Table 4.5. Relationship between CHD and lipid profile.

Lipid profiles	Mean			
	CHD group	Control	t -value	p-value
Cholesterol (mg\dl)	193.6	183.49	1.3	0.19
Triglyceride (mg\dl)	133.86	116.94	1.6	0.1
LDL (mg\dl)	126	111.47	2.3	0.02
HDL (mg\dl)	39.70	49.94	4.11	0.000

4.8. Relationship Between Lipid Profile and *ApoE* Genotypes.

The relationship between lipid profile and *ApoE* is provided in Table 4.6.
As shown in the table there was no significant difference between the mean of cholesterol, triglyceride and HDL regardless of the *ApoE* genotype. On the other hand, there is a significant difference between the *ApoE* genotype and the mean of LDL.

Table 4.6. Plasma lipid profile according to *ApoE* genotype

Lipid (mg\dl)	**Genotype**			
	E3/E3	E3/E4	E3/E2	p- value
T.C	184.52	218.17	149.67	0.8
T.G	128.36	128.31	95.33	0.3
LDL	112.94	149.31	93.67	0.02
HDL	45.66	43.97	37.08	0.2

4.9. Relationship Between Lipid Profile and Gender

The relationship between lipid profile and gender is provided in Table 4.7.
As shown in the table there was no significant difference between male and female in terms of their lipid profiles.

Table 4.7. Relationship between lipid profile and gender.

Lipid (mg\dl)	**Male**	**female**	**p-value**
Cholesterol	186.10	191.8	0.5
Triglyceride	128.36	121.63	0.2
LDL	118	120.20	0.7
HDL	42.53	47.22	0.06

4.10. Relationship Between *ApoE* Genotype and gender

The results showed that there is no statistically significant relation between ApoE genotype and gender (X^2 = 3.39, p-value= 0.18), Table 4.8.

Table 4.8. Relationship between *ApoE* gene and Gender.

Genotype	Male	Female	Total	p-value
E3/E3	50	46	96	**0.18**
E3/E4	19	10	29	
E3/E2	9	3	12	
Total	78	59	137	

CHAPTER 5
Discussion

CHAPTER 5
Discussion

ApoE is a polymorphic protein consisting of a single, 299 amino acids polypeptide chain. The three major isoforms of the protein are *ApoE2*, *ApoE3* and *ApoE4.* These differ from each other by cysteine-arginine substitutions at amino acid residues 112 and 158. These interchanges result from single-base changes of a thymine to a cytosine at the relevant position in the *ApoE* coding region. The biosynthesis of each protein isoform is under the control of three independent codominant alleles, E2, E3, and E4, located at a single *ApoE* gene locus on chromosome 19q13. Depending on the inheritance of any two alleles, six *ApoE* genotypes are possible. *ApoE* is an integral surface constituent of triglyceride-rich chylomicrons, chylomicron remnants and the lipoproteins VLDL and HDL, and acts in the receptor-mediated metabolism of these particles. The *ApoE* genotype alters risk predisposition to cardiovascular disease **(35)**.

The objectives of this study were to evaluate the effect of *ApoE* gene polymorphism on plasma lipid levels and to investigate the association between plasma lipid levels and CHD in Gaza Strip.

To our knowledge, this is the first study in Gaza Strip investigating the *ApoE* gene polymorphism in relation to lipid profile and CHD.

5.1. Genotype Frequencies of *ApoE* in Gaza Strip

According to the results obtained from PCR-RFLP the *ApoE* allele frequencies in the control group were: 0.0515 for the E2 allele, 0.875 for the E3 allele and 0.073 for the E4 allele. This distribution is in agreement with the distribution with the control group reported in a Saudi study were they recorded: 0.888 for E3, 0.050 for E2, and 0.062 for E4 **(16)**.

The frequencies of the *ApoE* alleles in the CHD subjects were: 0.0362 for the E2 allele, 0.826 for the E3 allele and 0.1377 for the E4.These frequencies are

also comparable to those of the Saudi study where they found 0.047 for the E2 allele, 0.849 for the E3 allele and 0.104 for the E4 allele **(16)**.

Our results showed that the distribution of the *ApoE* genotypes in Gaza Strip is similar to that of other Asian and Saudi populations as shown in Table 5.1 which represents the distribution of *ApoE* alleles in different human populations.

Table 5.1. Distribution of ApoE alleles in different human populations.

Population	E2	E3	E4	Reference
Caucasians (Tyroleans)	0.090	0.789	0.117	**(36)**
Blacks (Khoi San)	0.077	0.553	0.370	**(37)**
Asians (Chinese)	0.076	0.875	0.049	**(38)**
Alaskans	0.020	0.787	0.193	**(39)**
Amazonian Amerindians	0.000	0.831	0.169	**(40)**
Saudi	0.0.050	0.888	0.062	**(16)**
Palestinians (Gaza Strip)	0.0515	0.875	0.073	(present study)

5.2. Relation Between *ApoE* gene Polymorphism and CHD

According to our results it was found from cases of the CHD that (19/69, 27.5%) had E3/E4 and from control group (10/68, 14.7%) . When compared to other studies, this percentage is close to that reported by **Dzimiri and Meyer (1999)** in Saudi Arabia where 20.8% of the CHD subjects had *E3/E4* as compared to 12.5% from control group**. Kolovou (2002)** has shown that 21.8% of CHD and 16.3 % of control group had the *E3/E4* genotype **(16, 41).** The present study however, indicated that there is no statistically significant relation between CHD and *ApoE* genes (X^2 = 3.4, p-value= 0.17). These results are in agreement with different studies such as the Saudi (1999) and the Taiwanese (2002) studies which recorded that no statistical difference in

genotype or allelic distribution was found between the patients of CHD and the control groups **(16, 41).** These results can be explained by the fact that other genetic or environmental risk factors may interact with *ApoE*4 in determining the CHD risk. We did not find a significant difference for the *Apo E* gene and genotype frequencies between patients and controls, suggesting that the E4 allele is not a strong risk factor alone for CHD in Gaza Strip. For the E4-allele, a significantly increased frequency in patients compared to healthy controls has been described in some but not all studies and the E4-allele could be a risk factor in association with other genetic or environmental factors **(42)**.

Cardiovascular disease (CVD) risk is influenced by several well-established risk factors, such as body mass index (BMI), an indicator of overweight and obesity, blood lipids, diabetes, and blood pressure. These are intermediate phenotypes, correlated among themselves and having their own genetic and environmental determinants, including diet, nutrition, hormones, smoking, alcohol intake, and physical activity **(43, 44)**

Studies in humans and mice indicate that both the type and quantity of blood-borne lipids are predictive of cardiovascular health or disease and that a relatively large number of proteins are involved directly or indirectly in the transport, maintenance and elimination of blood lipids, including (HDL-C and LDL-C, respectively). Eleven of these proteins with a well-known collective activity in the reverse transport of cholesterol from peripheral cells to the liver have been identified. Genes coding for these proteins that have been implicated with blood lipids comprise: ATP-binding cassette protein 1 (ABCA1) ; apolipoprotein A1 (*ApoA1*); *ApoE* ; cholesterol-ester transfer protein (CETP) endothelial lipase (EL) ; hepatic lipase (HL) **(45, 46, 47)**; lecithin–cholesterol acetyl transferase (LCAT); low density lipoprotein receptor (LDLR) ; lipoprotein lipase (LPL); phospholipid transfer protein (PLTP); and scavenger receptor class B type I (SR-BI). Consequently, variation in one or more of these genes can be important as a risk factor in developing CHD **(48, 49, 50).**

5.3. Relation between Smoking and CHD

Cigarette smoking is an important and reversible risk factor for CHD. Incidence of myocardial infarction is increased six fold in women and three fold in men who smoke at least 20 cigarettes per day compared to subjects who never smoked. The risk increases with tobacco users in both men and women and is higher in inhalers compared to non inhalers **(51, 52).**

Our results indicated that there is a statistically significant difference between the smokers and non-smokers in the case and control groups (X^2= 4.56, P-value= 0.049) where 69.2% of CHD subjects are smokers, as compared to the control subjects where 38.8% of them are smokers. This result further supports the notion that smoking is one of the risk factors of CHD.

The study of **Steve et al. (2001)** has shown that the risk of CHD among smokers was 1.94 higher than in never-smokers. *ApoE* genotype had no effect on CHD risk among never-smokers, but it had a great effect in smokers. Among smokers, the adjusted hazard ratio for CHD was 1.47 for men with the E3 allele, 0.85 for those with the E2 allele, and 2.79 for carriers of the E4 variant, compared with never-smokers **(53).**

Smoking is known to increase the risk of atherosclerosis by boosting oxidation of cholesterol, ApoE seems to be important in preventing this oxidation process and while the E4 version is a very poor antioxidant, E3 seem to be better in preventing oxidation of cholesterol. This fact leads us to the view that certain alleles of ApoE play an important role in protecting cholesterol and with it, plaque formation **(53).**

It is estimated that exposure to second hand smoke (passive smoking) causes almost 40000 deaths from heart disease each year in the United States **(51),** and increases the risk of coronary disease and coronary death by approximately 20% **(52).**

In our study we found that T.G is significantly higher in smokers as compared to non-smokers. Moreover, HDL was significantly lower in smokers. These results support previous findings suggesting that smoking is associated with

adverse effects on serum lipids which include elevation of TG and reduction of HDL **(54).**

Steve *et al* (2001) have shown that the effect of smoking is independent of *ApoE* polymorphism on cholesterol levels. This means that, regardless of your cholesterol level, if you have the E4 version and smoke, the risk is high **(53).**

In our study we found that 9 patients of CHD have alleles E4 and smokers on the other hand in the control group there is no persons having alleles E4 who are smokers.

5.4. Relation between gender and CHD

It has been recognized for many years that men are at higher risk for developing CHD than women. The male-to-female ratio for fatal CHD is consistently around 2:1 in various countries with different lifestyles and rates of heart disease. The sex difference in CHD incidence persists after taking into account common CHD risk factors **(54).** CHD is rare among premenopausal women, and the Framingham Study revealed that the incidence rate of CHD in postmenopausal women is more than double that in premenopausal women of the same age. These findings have led to the hypothesis that endogenous sex hormones may play a role in CHD in men and women. In particular, it has been postulated that estrogens provide protection against CHD **(55).**

CHD is markedly more common in men than in women. In both sexes, CHD risk increases with age, but the increase is sharper in women **(56).**

Sex hormones are known to influence enzymatic activities, with hepatic lipase being stimulated by testosterone **(57).** Thus, the magnitude of the sex differences in levels of LDL, VLDL, and HDL by *ApoE* genotype may very well be determined by the modulating effects of hormones on the processes that control these lipoproteins. In premenopausal women, the effect of estrogen on lowering LDL may influence the effects of the *ApoE* genotype. Conversely, the absence of estrogen in postmenopausal women raises VLDL concentrations, which may influence the effects of *ApoE* on VLDL metabolism. In our study we

showed that the percentage of CHD in males is higher than that in females and one explanation of this result may be the advanced age of the women (postmenopausal) where the absence of estrogen raises LDL**.**

5.5. Relation between age and CHD

As we noted from our results, advanced age increases the risk of CHD where the mean age of cases was 57.65 years. This observation is in agreement with the fact that advancing age is one risk factor used as a surrogate for atherosclerotic plaque burden. With aging there is a gradual but progressive accumulation of coronary plaques. This accounts for the increasing risk of CHD with advancing age **(58).**

5.6. Serum Lipid Profile and the *ApoE* Genotypes

As we noted from our results the mean of cholesterol level (218.17mg\dl) is elevated in subjects with E4 genotype as compared to those with E3 (184.5 mg/dl), while the E2 genotype has the lowest level (149.6 mg/dl). These results confirm the effect of variation in *ApoE* genotype on lipoprotein levels. The E4 allele has been shown to increase intestinal cholesterol absorption, to affect LDL synthesis in the liver, and to be associated with elevated levels of total cholesterol and LDL-C and a higher prevalence of atherosclerosis. The association between *ApoE* polymorphism and CHD has been found in several cross-sectional studies **(59).**

Subjects with different *ApoE* genotypes differ in the absorption efficiency of cholesterol from the intestine, in the synthesis rates of cholesterol and bile acids, and in the production of LDL apoprotein B. This suggests that the response of serum cholesterol to diet may be affected by the *Apo* E2/E3/E4 polymorphism **(60, 61).**

It is important to note here that in our results the LDL was significantly increased in ApoE4 and decreased in *ApoE2*. *ApoE2* exhibits lower affinity for

the LDL receptor, resulting in slower clearance of *ApoE* and higher plasma *ApoE l*evels **(62).** In response, the liver up-regulates the LDL receptor, resulting in lower cholesterol levels. Conversely, *ApoE4* is cleared more efficiently, resulting in lower ApoE levels and higher cholesterol levels. The genetic variations thus affect lipid metabolism and have been shown to alter risk of cardiovascular disease **(63, 64).**

In the general population, the E2 allele is consistently associated with lower levels of total plasma cholesterol, LDL cholesterol, and *Apo B* and elevated levels of TG and ApoE compared with the E3 allele. Elevated levels of TG and ApoE are consistent with impaired clearance of remnant particles containing *ApoE2*, presumably due to defective receptor recognition of *ApoE2* containing particles **(65, 66).** The basis for the reduced *ApoB* and LDL cholesterol levels in *ApoE2/3* and 2/2 individuals is less clear. **Ehnholm *et al* (1984)** suggested that the presence of *ApoE2* in intestinal VLDL particles impairs their conversion to LDL by interfering with normal lipolytic processing. Conversely, the E4 allele is associated with higher levels of total and LDL cholesterol and Apo B and lower levels of ApoE **(14).** These observations are consistent with the faster rate of catabolism of particles containing ApoE4 compared with those containing *ApoE3*. Affinity for LDL receptor is lower for *ApoE2* protein and higher for the *ApoE4* variant, while *ApoE3* shows normal binding activity. Therefore, *ApoE2* and *ApoE4* alleles are associated with high TG and high TC, respectively **(67, 68).**

5.7. CHD and Lipid Profile

Our study results showed that there is a significant increase in LDL in the case group as compared to the control group. Many authors have shown similar results that increases in LDL are correlated with a high CHD risk **(56).**

In the present study no statistically significant differences were evident between the mean of chololestrol between the case group and the control group. It has been suggested that LDL-C indicates cardiovascular risk in

individuals better than total cholesterol. This result is in agreement with that reported by other authors the most common lipid disorder was high TC and high LDL-C in CHD, which was much more prevalent than high TG

A high concentration of LDL must be considered the principle risk factor because in the absence of elevation of LDL-C atherosclerosis develops slowly and usually remains subclinical. This is true even when the other independent risk factors are present **(69).**

Our study results indicated that there is no significant difference between the mean of cholesterol in CHD group and the control group. There are several possible explanations for the absence of a positive association between total cholesterol and CHD. First, the total cholesterol level in the elderly may not represent their lifetime exposure, because many lifestyle factors and diseases may modulate it with advancing age **(58).** Thus, total cholesterol values measured in old age may differ considerably from levels determined by genetic and dietary factors in young and middle age. Second, cholesterol levels decline with age and with chronic disease, inflammation, malnutrition, or poor health status in elderly persons. Thus, lower cholesterol levels in the elderly may represent a surrogate for comorbidity, frailty, or subclinical disease. Although some studies found that elevated total cholesterol levels have been related to mortality from CHD after adjustment for frailty or comorbidity and cardiovascular risk factors such as stroke, serum albumin, and iron, other studies have found that low cholesterol is related to mortality even with adjustment for health status and indicators of frailty **(70)**

In the present study, however, no association between triglyceride and CHD was found but there is association between the HDL and CHD and this result is in agreement with a recent study which provides evidence that low HDL-C is associated with increased CHD mortality in older age groups. HDL-C has emerged as an important independent predictor of CAD. Every 1mg/dl decrease in HDL-C causes a 3-4% increase in CHD **(70).** Fasting triglyceride

level represents a useful marker of risk for CAD, especially when HDL-levels are considered. All patients with low HDL and/or high triglyceride should receive recommendations for life style modifications that include diet low in saturated fat and increased physical activity.

CHAPTER 6
CONCLUSION and RECOMMENDATIONS

CHAPTER 6
CONCLUSION and RECOMMENDATIONS

The present study focused on detection of *ApoE* genotypes in Gaza Strip and the relationship between those genotypes and lipid profile in 69 cases of CHD from Nasser and AlShifa hospitals as compared to 68 healthy subjects. The results of this study can be summarized as follows:

- In Gaza Strip, the *ApoE3/E3* genotype was the most common in the control and the CHD groups. ApoE2/E3 and *ApoE4/E3* were the next most common genotypes. Other genotypes such as *ApoE2/E2*, *ApoE4/E4*, or *ApoE2/E4* were not encountered in the examined subjects.
- The frequencies of *ApoE* alleles in the CHD subjects were: 0.826 for the E3, 0.137 for the E4 and 0.0362 for the E2. These frequencies are comparable to those found in the control group where we obtained: 0.875 for the E3, 0.073 for the E4 and 0.0515 for the E2.
- No statistically significant differences in *ApoE* genotypes were found between the patients and the control groups.
- The distribution of the *ApoE* genotypes in the Gaza Strip population is similar to that of other Asian populations.
- There is no significant difference between male and female in terms of the *ApoE* genotypes
- Our study indicated that there is no significant difference between the mean of cholesterol and triglyceride levels of the CHD and the control groups.
- There is a significant difference between the mean of LDL and HDL levels between the CHD and the control group.
- There is no significant difference between male and female in lipid profile in old age.

- There is no significant difference between the mean of triglyceride and HDL between the different *ApoE* genotypes. However, there is a significant difference in the mean of LDL and *ApoE* genotypes.
- Smoking, hypertension and advanced age are important risk factors for CHD.

To our knowledge, this is the first study in Gaza Strip investigating the relation between ApoE genotypes and CHD. Based on the data presented by this study, further studies are therefore needed to address the impact of variation of the *ApoE* locus on plasma lipid fractions and risk of atherosclerosis.

Finally, CHD is still a health problem, particularly in old age, not only in Gaza Strip, but worldwide, so variations in other relevant genes should be addressed and further investigations are needed to link other genetic factors to CHD.

CHAPTER 7
References

CHAPTER 7
References

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Appendix A

الجـــــــــامعة الاســـــــلامية – غزة
The Islamic University of Gaza
رئيس قسم الأحياء
برنامج ماجستير العلوم الحياتية

كلية العلوم

التاريخ/ 23/04/2005

الأخ د. فيصل أبو شهلا
مدير عام المستشفيات، حفظه الله،

السلام عليكم ورحمة الله وبركاته،

الموضوع/ تسهيل مهمة باحث

نود أن نعلم سيادتكم بأن الطالبة لمياء أبو مرزوق تقوم بإجراء البحث العملي الخاص بها وذلك ضمن برنامج ماجستير العلوم الحياتية تخصص تحاليل طبية، وبحثها بعنوان:-

Association between *apoE* gene Polymorphism and Serum lipid Profile in Coronary Heart Disease and Healthy Subjects.

لذا نرجو من سيادتكم تسهيل مهمة الطالبة في جمع العينات اللازمة لبحثها.
سيجرى البحث في بداية يونيو 2005 على عينات من مرضى القلب تجمع من مستشفى الشفاء ومستشفى ناصر.

ولكم منا جزيل الشكر.

رئيس قسم الأحياء
مدير برنامج الماجستير
د. عبود القيشاوي

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الجامعة الإسلامية-غزة – الرمال ص.ب:108 فلسطين

Appendix B

بسم الله الرحمن الرحيم

رقم الاستبيان ( )

نتوجه إليكم بجزيل الشكر والامتنان لتعاونكم معنا في إنجاز بحث بعنوان "ما مدى الارتباط بين أشكال الجين apoE ومستوى الدهون في كل من مرضى القلب والأشخاص الأصحاء" ،علماً بأن جميع المعلومات التي ستكون في هذا الاستبيان سرية جداً.

وشكرا لحسن تعاونكم،،،

مقدمة البحث

- الاسم: .....
- الجنس ( ) ذكر ( ) أنثى
- العمر.....
- مكان السكن ( ) المنطقة الشمالية ( ) المنطقة الوسطى ( ) المنطقة الجنوبية
- مستوى التعليم ( ) دراسات عليا ( ) جامعة ( ) ثانوية عامة ( ) دون
- هل لديك مهنة ( ) نعم ( )لا
- طبيعة المهنة .....

* هل تمارس أي نوع من أنواع الرياضة

( ) نعم ( )لا

* هل أنت مدخن

( ) نعم ( )لا

* هل أنت مريض سكر

( ) نعم ( )لا

* هل تعاني من ارتفاع في ضغط الدم

( ) نعم ( )لا

* هل تعاني من أي مشاكل في القلب

( ) نعم ( ) لا

* هل هناك أشخاص في العائلة يعانون من أمراض في قلب

( ) نعم ( ) لا

* ما هو مستوى الدهون لديك

( ) طبيعي ( ) مرتفع ( ) لا أعرف

* هل تأخذ علاج لتعديل مستوى الدهون

( ) نعم ( ) لا

وشكرا لحسن تعاونكم،،،

مقدمة البحث

لمياء فيصل ابومرزوق

Appendix C

بسم الله الرحمن الرحيم

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

Date: 14/6/2005

التاريخ: 2005/6/14

Mrs./ Lamia Abu Marrzoq

السيدة: لمياء أبو مرزوق

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

Association between apoE gene polymorphism and serum lipid profile in coronary heart disease and healthy subjects.

In its meeting on June 2005

و ذلك في جلستها المنعقدة لشهر يونيو 2005

and decided the Following:-

و قد قررت ما يلي:-

To approve the above mention research study.

الموافقة على البحث المذكور عاليه.

على أن يتم الحصول على الموافقة الخطية من المرضى المراد إجراء البحث عليهم وتوضيح أهداف البحث العلمي وأن لهم الحق في الانسحاب من البحث في أي وقت يشاءون ذلك

Signature

توقيع

Member

عضو

Member

عضو

Chairperson

Ministry of Health
Helsinki Committee

Conditions:-

- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciate receiving one copy of your final research when it is completed.

Gaza Etwam – Telefax 972-7-2878166